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Imprinting of mouse *Kvlqt1* is developmentally regulated

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Mouse distal chromosome 7 contains a cluster of at least five imprinted genes. The syntenic region in humans, at 11p15.5, has been implicated in several genetic disorders. Consistent with the imprinted status of the genes in the region, Beckwith–Wiedemann syndrome (BWS) and Wilms tumor are each associated with loss of maternal information. Also mapping to 11p15.5 are long QT and Jervell and Lange-Nielsen (JLN) syndromes. In contrast to BWS and Wilms tumor, these syndromes do not show any parent of origin bias. Recently positional cloning has identified *KVLQT1* as the 11p15.5 gene responsible for increased susceptibility to long QT and JLN syndromes. Other studies associate *KVLQT1* with BWS. Human *KVLQT1* is paternally imprinted in embryos. In this study we present a contig and transcript map of distal mouse 7 and we physically and genetically map mouse *Kvlqt1* to the region. Mouse *Kvlqt1* is strongly expressed in heart, lung, gut, kidney and uterus. While its early developmental expression is maternal in origin, the paternal allele becomes increasingly active during development. Late juvenile and adult animals show complete biallelism, suggesting an explanation for the lack of parent of origin bias in JLN and long QT.

INTRODUCTION

Genomic imprinting is an epigenetic mechanism controlling gene expression in which the transcriptional activity of each allele is dependent on its parental origin. More than 20 genes have been demonstrated to show imprinting in mice and humans. Many imprinted genes map to discrete clusters in the mammalian genome. However, the functional significance of the clustering is not yet clear (1).

One cluster of imprinted genes is at the distal end of mouse chromosome 7. Five imprinted genes have previously been localized to this region (Fig. 1). Uniparental disomies of distal 7 result in embryonic lethality, demonstrating the importance of maintaining the appropriate gene dosages of these genes (2,3). The syntenic region in humans, at 11p15.5, has been implicated in several genetic disorders. Beckwith–Wiedemann syndrome (BWS), a childhood overgrowth syndrome, and Wilms tumor (WT) are each associated with loss of maternal information, consistent with the imprinted status of the genes in the cluster (4). Also mapping to 11p15.5 are long QT and Jervell and Lange-Nielsen (JLN) syndromes (5,6). Long QT syndrome is characterized by cardiac arrhythmias and an extended QT interval on electrocardiograms, while JLN is characterized by deafness in addition to the cardiac abnormalities observed in long QT (7,8). In contrast to BWS and WT, these syndromes do not show any parent of origin bias (5,7). In addition, while long QT syndrome is inherited as a dominant trait, the JLN syndrome is a recessive phenotype. The gene increasing susceptibility to JLN is thus expected to be biallelically expressed, at least in tissues affected by the mutation.

Recently, positional cloning has identified *KVLQT1* as the 11p15.5 gene responsible for long QT and JLN syndromes (9). *KVLQT1* encodes a protein which dimerizes with minK to produce a functional potassium channel (10–12). Mutations in this gene cause susceptibility to long QT and to JLN syndromes (5,9). In addition to its role in long QT syndrome, human *KVLQT1* was recently isolated in a search for genes associated with BWS. *KVLQT1* is linked to BWS in that the gene spans translocation breakpoints associated with the disease (13). With some exceptions *KVLQT1* is paternally imprinted in embryos, i.e. the paternally derived allele is silent (13).

In this study we present a contig and a transcript map of mouse distal 7 and we genetically and physically map the mouse *Kvlqt1* gene to distal 7. Mouse *Kvlqt1* is strongly expressed in heart, lung, kidney, gut and uterus. While its early expression is maternal in origin, the paternal allele becomes increasingly active during development. In lung, kidney and gut the paternal allele is fully active in late juveniles and adults. In cardiac tissue activation of the paternal allele is seen in late embryogenesis. Juvenile and adult animals show complete biallelism, suggesting an explanation for the lack of parent of origin bias in JLN and long QT.

RESULTS

To develop models for the human disorders and to assist in understanding the molecular basis of genomic imprinting we are interested in constructing a complete physical and transcript map of the mouse distal 7 cluster. Starting at *p57Kip2*, *Mash2*, *Igf-2* and *H19*, we first identified bacterial artificial chromosomes (BACs) through PCR and hybridization screens (Fig. 1).

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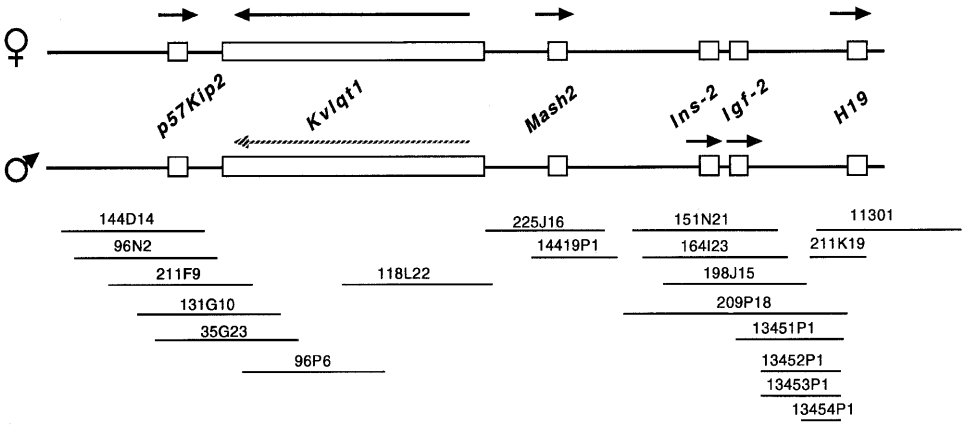

Figure 1. Transcript map of the mouse distal chromosome 7 imprinted cluster. Transcription from maternal and paternal chromosomes is indicated by the arrows. *H19* (15), *Mash2* (16) and *p57Kip2* (17) are all expressed from the maternal chromosome, while *Ins-2* (18) and *Igf-2* (19) transcripts are paternal in origin. The direction of transcription is indicated by the arrowhead. *Kvlqt1* spans a distance of at least 250 kb. A physical map of overlapping BACs and P1s spanning the region is shown. P1 clone names all end with the designation P1. 144D14, 96N2, 211F9, 131G10 and 35G23 were obtained using *p57Kip2*-specific PCR primers. 96P6 was obtained by walking from the SP6 end of 35G23. 225J16 was obtained using *Mash2*-specific PCR primers. 118L22 and 14419P1 were obtained by walking from the T7 and the SP6 ends of 225J16 respectively. 151N21, 164I23 and 198J15 were obtained using *Igf-2*-specific PCR primers. 13451P1, 13452P1, 13453P1 and 13454P1 clones were obtained using H19 5′ imprinting box-specific primers (20). 113O1 was obtained using a DNA probe containing *H19* promoter sequences. 211K19 and 209P18 were obtained by walking from the T7 end of 113O1. BAC clones range in size from 90 to 140 kb.

Overlaps between clones were established by end-fragment hybridization. By chromosome walking we identified additional BACs and P1s which bridged the distances. Using probes derived from our BAC clones we demonstrated that transcription of *Mash2* and of *p57Kip2* is from the same DNA template strand as *H19*, *Igf-2* and *Ins-2* (Fig. 1) (see Materials and Methods).

Direct sequencing of BAC188L22 revealed homology to exon 9 of human *KVLQT1*. BAC188L22 was isolated in our walk starting from *Mash2*. Furthermore, we have determined that *Kvlqt1* sequences are present on overlapping BACs isolated independently using *p57Kip2* PCR primers (Fig. 1). Together, this provides strong support that *Kvlqt1* is physically linked with these genes. To confirm the linkage genetically we identified an *Nla*III polymorphism at the 3′-end of *Kvlqt1* that distinguishes *Mus castaneus* and *Mus domesticus* alleles. Thirty one interstrain backcrosses revealed zero crossovers between *Kvlqt1*, *H19* and other genetic markers in the region (data not shown; see Materials and Methods). As described in Materials and Methods, we determined that transcription of mouse *Kvlqt1* is in the opposite orientation relative to other genes in the cluster.

To begin to understand regulation and function of the gene in mouse development we first analyzed expression of the RNA by northern blotting. In addition to the expression in heart, salivary gland and kidney previously reported for mouse *Kvlqt1* (10), we have noted high levels of expression of the ~3.2 kb transcript in gut, lung, testes and uterus (Fig. 2A). Expression is noted early during organogenesis and remains relatively constant throughout development. Adult levels of mRNA are detected by northern blot in e12.5 heart (Fig. 2B) and in e13.5 lung (data not shown), the earliest developmental stages assayed for specific expression in these tissues. In kidney and gut low levels of expression were noted at e13.5, while high levels of expression were reached by e16.5 (data not shown).

Because human *KVLQT1* and other genes in the cluster are imprinted, we developed an assay to distinguish between maternal and paternal expression of the mouse gene. We

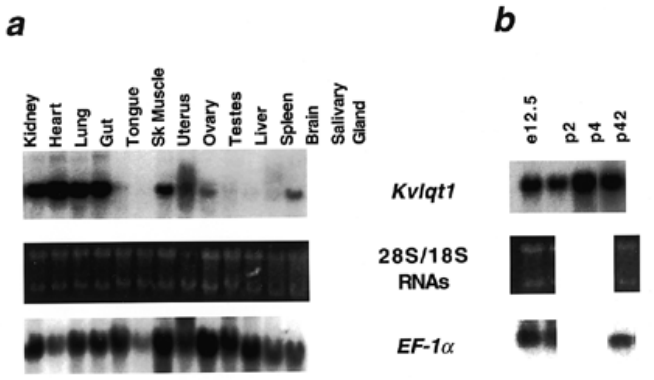

Figure 2. Tissue and developmental expression patterns of mouse *Kvlqt1*. (**A**) Northern analyses of 10 μg total RNA revealed a 3.2 kb *Kvlqt1* transcript in adult mouse kidney, heart, lung, gut and uterus (top). Lower levels are noted in salivary gland, testes and ovary. A minimum of two samples from adult and neonatal mice were analyzed. Representative results for adult tissues are displayed. (**B**) Northern analysis of the developmental regulation of *Kvlqt1* expression. Five micrograms of total RNA from e12.5, neonatal and adult hearts were analyzed. *Kvlqt1* RNA levels were normalized by staining with ethidium bromide for total RNA (middle) and probing stripped blots for elongation factor-1a (*EF-1*a) (lower).

examined expression in progeny of crosses between *M.domesticus* and a congenic strain in which distal chromosome 7 is *M.castaneous* in origin. Crosses were performed so that the maternal allele was *domesticus* in origin. cDNA was amplified and *castaneus* and *domesticus* alleles were distinguished using the same *Nla*III polymorphism as used for the genetic mapping (Fig. 3A). Examples of results for adult progeny are shown in Figure 3B for gut, kidney, heart and lung. Expression is clearly biallelic, with comparable levels of expression from the maternal and paternal alleles. Similar results were noted with early

neonates (p2.5 and p0.5) (data not shown). To insure that imprinting was occurring normally in progeny of our congenic strain we tested for imprinting of the well-characterized gene *H19* (14,15), using RT-PCR and a *Cvi*JI polymorphism. As expected, only maternally derived transcripts were detected from both *domesticus* × *castaneus* and from *castaneus* × *domesticus* crosses (Fig. 4).

To compare our results more directly with those noted previously in humans (13) we examined imprinting in RNAs obtained from e18.5, e16.5, e13.5 and e12.5 tissues and from RNA derived from total e8.5 embryos. Samples of these analyses shown in Figure 3C indicate that imprinting of *Kvlqt1* is under developmental regulation, with a strong maternal bias in early embryos that in a tissue-specific manner is gradually lost as embryogenesis proceeds. Expression in the heart is biallelic by e16.5, while in lung, gut and kidney fully biallelic expression is not noted until p0.5 (data not shown). In contrast, expression in yolk sac is biallelic at e13.5 (Fig. 3C) and at e12.5 (data not shown). We have not looked earlier in development to determine if expression in this extra-embryonic tissue begins with a maternal bias.

We assumed that monoallelic expression of *Kvlqt1* in embryonic tissues was due to imprinting. However, an alternative explanation is that *castaneus* promoter and/or enhancer elements show low levels of transcriptional activity in the fetus relative to the *domesticus* regulatory elements independent of parental origin. To distinguish between these two possibilities we analyzed RNAs from progeny in which the maternal allele was of *castaneus* origin. As previously noted (Fig. 3C), transcription in e13.5 embryos shows a very strong maternal bias in all tissues examined, while expression in adult tissues is biallelic, with approximately equal contributions from the maternal and paternal chromosomes (Fig. 3D). These results thus confirm that expression of *Kvlqt1* is subject to genomic imprinting and that the imprinting is under developmental control. We did note one significant difference in RNA expression patterns from the reciprocal crosses. Fully biallelic expression in *castaneus* × *domesticus* crosses is delayed relative to that seen in *domesticus* × *castaneus* crosses. Expression in the heart is biallelic by p0.5 (compared with e16.5, Fig. 3C), while paternal expression in kidney, gut and lung is not maximum until 12 days post-natal (data not shown).

Finally, we wished to confirm that the maternal bias noted in very early embryos was due to imprinting and not to contamination of our dissected fetuses with maternal tissue. Therefore, blastocysts from *castaneus* × *domesticus* crosses were transferred to domesticus foster mothers for development to e9.5. RNA analysis results were identical to those seen in the last panel of Figure 3C.

DISCUSSION

In conclusion, we mapped the mouse *Kvlqt1* gene to contiguous BAC clones from the imprinted cluster of mouse distal 7 and have genetically mapped the gene to the same region. Mouse *Kvlqt1* is a large gene, spanning at least 250 kb. Its transcription is in the opposite orientation to all other known mouse genes in the cluster. *Kvlqt1* shows expression patterns suggesting that it is not limited in function to repolarization of cardiac cells.

Initial expression of *Kvlqt1* is primarily from the maternal allele, but by late embryogenesis there are significant contribu-

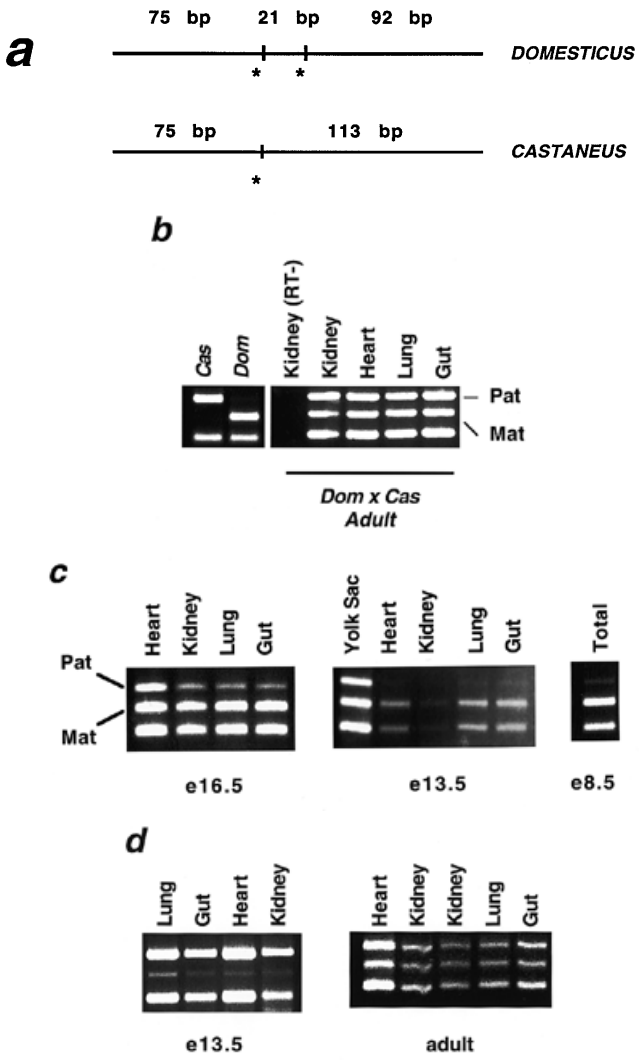

Figure 3. Developmental regulation of imprinting of *Kvlqt1*. (**A**) Primers spanning a 3′ intron were used to generate a 188 bp product from cDNA. The positions of two *Nla*III sites are indicated by asterisks (*). One site is found only in the *domesticus* PCR product. Thus the 113 and 92 bp *Nla*III digestion products mark the *domesticus* and *castaneus* alleles respectively, while a 75 bp fragment is common to both alleles. Template mixing experiments indicate that we can detect transcription from one allele when it is 5% of the total *Kvlqt1* mRNA. These experiments also suggest a slight bias toward the *castaneus* allele. (**B**) Biallelic expression of *Kvlqt1* in adult tissues of *domesticus* × *castaneus* progeny. cDNA was synthesized from 1–2 µg total RNA from kidney, heart, lung and gut and then amplified with primers specific to the 3′-end of *Kvlqt1*. These primers span an intron and therefore do not amplify genomic DNA. Amplified DNA was digested with *Nla*III and analyzed by gel electrophoresis. The digestion products for *castaneus* and *domesticus* RNA controls are shown in the first two lanes. The maternal (Mat)- and paternal (Pat)-specific products are indicated. Multiple RNA samples for each age and tissue type were analyzed and typical results are displayed. Each RNA sample was processed with and without reverse transcriptase (RT–). All RT– controls were analyzed by gel electrophoresis; however, only one example of this control is displayed. (**C**) Allele-specific expression of *Kvlqt1* in embryonic tissues prepared from progeny of *domesticus* × *castaneus* crosses. RNAs from at least three individual embryos were analyzed as described in (B) and representative results displayed. For e13.5 kidney and for e8.5 total embryos multiple samples were pooled for each RNA extraction. Lanes are marked as in (B). (**D**) Allele-specific expression of *Kvlqt1* in embryonic tissues prepared from progeny of *castaneus* × *domesticus* crosses. RNAs from at least three progeny were analyzed as described in (B) and representative results displayed for e13.5 embryos and 5-week-old adults.

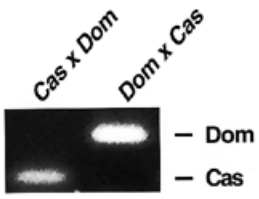

Figure 4. Maternal-specific expression of *H19* in neonatal kidney. cDNAs from neonatal kidneys were amplified using primers specific to exon 5 of the mouse *H19* gene. The 163 bp product was digested with *Cvi*JI and analyzed by gel electrophoresis. Seventy base pair *castaneus*-specific and 110 bp *domesticus*-specific fragments were generated. The genotypes of the assayed samples are indicated above the gel.

tions from the paternal allele. In adult mice there is no obvious difference in expression between the two alleles. Genomic imprinting of *KVLQT1* has previously been noted in human samples (13). In multiple fetal lung and kidney samples expression was consistently monoallelic. In fetal heart two samples showed monoallelic expression while four samples showed biallelic expression with a bias towards one allele. These mouse results showing developmental and tissue-specific regulation of *Kvlqt1* imprinting are consistent with the reported human results. This developmentally regulated loss of imprinting, if conserved in humans, suggests an explanation for the heterogeneity noted in human fetal heart expression and also for the lack of parent of origin bias in inheritance of the two syndromes linked to this gene, JLN and long QT.

The mechanism for loss of imprinting in post-natal mice is not yet clear. We have considered two possibilities. Activation of the paternal allele may be due to recruitment of alternate promoters that are not subject to genomic imprinting. Consistent with results for human *KVLQT1* (13), we have identified multiple isoforms of the mouse gene. These include transcripts specific to heart, to gut and to lung and kidney as well as transcripts common to all these tissues. However, we have no evidence for developmental induction of these transcripts. Therefore, we currently favor the notion that loss of imprinting is due to loss of paternal-specific repression of promoters already active on the maternal chromosome. Experiments to develop assays that can distinguish between these isoforms as well as determine parental origin are currently underway.

Expression of the two *Kvlqt1* alleles is clearly effected by parental origin: mouse *Kvlqt1* is imprinted. However, the outcome of the imprint is certainly qualitatively different from that noted for some other imprinted genes, where expression from the imprinted allele is completely absent (see Fig. 4 for example). Rather, the effect of the imprint at mouse *Kvlqt1* is quantitative. In conjunction with the complex tissue and developmental regulation of silencing now noted for several imprinted genes, these results suggest that the consequences of allelic differences or mutations in imprinted genes on development and disease may be interestingly complex.

MATERIALS AND METHODS

Contig identification

All BAC and P1 clones were obtained by screening libraries supplied by Genome Systems Inc. Distal ends of the mouse genomic clones were obtained by direct cycle sequencing. Oligonucleotide probes were used to align BACs. PCR primers were then used to walk from the furthest extending BAC. BAC sizes were determined by pulsed field gel electrophoresis.

Genetic mapping

DNA from F_1 backcross progeny of *M.domesticus* (FVB) × *M.castaneus* crosses were genotyped for *Kvlqt1*, *H19*, D7Mit262 and D7Mit291. To genotype for *Kvlqt1* primers 5′-GGA CCA GAG ACT GGT GAT CAT C and 5′-TTG CTG GGT AGG AAG AGC TCA G were used to amplify DNA that upon digestion with *Nla*III yielded 92 and 113 bp fragments specific to *domesticus* and *castaneus* respectively. *H19* was genotyped as previously described (21).

Transcriptional orientation of *p57Kip2*, *Kvlqt1* and *Mash2*

Kvlqt1 was oriented by the distinct hybridization patterns of 5′- and 3′-specific *Kvlqt1* cDNA probes to BACs 225J16, 118L22 and 35G23. Probes specific to the 3′- and 5′-ends of *Mash2* (22) and *p57Kip2* (23) were obtained by subcloning BACs 35G23 and 225J16. *Mash2* was oriented by hybridization of a probe specific to 3′ sequences of that gene to the *H19* end of BAC 225J16. *p57Kip2* was oriented by hybridization of a 3′-specific *p57Kip2* probe with the *H19* end of BAC144D14.

Isolation of total RNA and northern blot analysis

Total RNA was isolated using TRIZOL (Gibco) and quantitated by absorbance at 260 nm. Quantitation and quality of the RNA was authenticated by assaying 1 µg by agarose gel electrophoresis and ethidium bromide staining. Up to 10 µg total RNA were assayed by northern blot analysis using an 1150 bp cDNA probe from the 3′-end of *Kvlqt1*. Blots were rehybridized with a 1100 bp elongation factor-1a probe to control for sample loading.

RT-PCR

cDNA was prepared using the Gibco Superscript Preamplification Kit and 1–2 µg total RNA. PCR products were amplified from cDNA using primers 5′-CAT CGG TGC CCG TCT GAA CAG G and 5′-TTG CTG GGT AGG AAG AGC CAG, which spanned intron 13 (using human *KVLQT1* nomenclature) (13). Reverse transcriptase-negative reactions were run concomitant to all samples to be certain of no contamination. The enzyme *Nla*III was used to digest the resultant 188 bp PCR product into 92, 75 and 21 bp fragments (*domesticus*) or into 113 and 75 bp fragments (*castaneus*). Primers 5′-GCA CTA AGT CGA TTG CAC TGG and 5′-GCC TCA AGC ACA CGG CCA CA were used to amplify a 163 bp *H19*-specific product which when digested with the enzyme *Cvi*JI yielded products of 110 (*castaneus*) or 70 bp (*domesticus*). The *H19* primers do not cross any intron boundaries and thus give identically sized products for both RNA and DNA.

Congenic strain

Female F_1 progeny of an *M.domesticus* (FVB) × *M.castaneus* cross were backcrossed to *M.domesticus* males for three generations. At each generation the presence of *castaneus* alleles at distal 7 was assayed using the D7Mit markers described above. Male and female progeny of the F_3 cross were mated and mice homozygous for *castaneus* alleles at the D7Mit markers were

selected. These mice were interbred to found the congenic strain used in this study.

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